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Mosaic Fate Mapping of the Behavioral and the Muscular Defects Induced by a *Drosophila* Mutation, *abnormal proboscis extension reflex C* (*aper C*)

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ABSTRACT—Mosaic analysis was applied to investigate whether the muscle degeneration induced by an X-linked mutation *aperC* is myogenic or neurogenic. The *aperC* mutation causes a progressive loss of the proboscis extension reflex to sugar stimulus. This behavioral defect is associated with the degeneration of a particular pair of muscles, the rostral protractors. Mosaic flies with mutant and wild-type tissues were produced by using an unstable ring-X chromosome. Both the behavioral defect and the muscle degeneration were examined after scoring the genotypes of the external structures of the mosaic flies. The behavioral defect in the mosaics correlated with the degeneration of the muscles. Over a half of the mosaics showed normal phenotypes in both the behavior and the muscles. Since very few mosaics showed hemilateral abnormality, the focus of the *aperC* mutation seems therefore to be submissive to the wild-type focus. The focus for the behavioral defect was located in the ventro-anterior region of the blastoderm fate map. The primary cause of the muscle degeneration of the *aperC* mutation must presumably therefore lie in the brain, not in the muscles.

INTRODUCTION

Hereditary muscular dystrophy covers a wide spectrum of muscle diseases both in vertebrates [1] and in invertebrates [2, 3]. The ultimate causes of muscular dystrophy have not yet been traced. Extensive studies have tried to determine whether the primary cause of muscular dystrophy in vertebrates is myogenic or neurogenic. For instance, cross innervation in the parabiosis of normal and dystrophic mice [4–6] and muscle transplantation between nomal and dystrophic mice [7, 8] have been executed. The conclusions are diverse, however, and have led to difficulties in the interpretation of the results. To overcome these difficulties, the mutant and the normal tissues must be juxtaposed at a very early stage of development. Peterson [9, 10] made a chimera mouse which was derived from the embryonic cell aggregation of normal and dystrophic mice. His chimera mosaic analysis indicates that some entirely extramuscular factors are primarily responsible for the muscle degeneration.

In *Drosophila melanogaster*, genetic mosaics with mutant and wild-type tissues can be produced by using an unstable ring-X chromosome. The internal anatomical site (focus) at which a mutant gene exerts its primary effect can be located on the blastoderm fate map by a statistical examination of the correlation between the genotypes of the external structures and the mutant phenotypes of the mosaics [11].

A recessive mutation, *abnormal proboscis extension reflex C* (*aperC*), causes degeneration of a pair of muscles, the rostral protractors which exert the force required for the proboscis extension reflex

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(PER) [12]. No muscle degeneration was seen in the newly eclosed mutants. The mutant muscles degenerate at advanced ages of eclosion. The muscle degeneration is influenced by temperature [3] and by the use or disuse of the muscle [13].

In this study, we applied the mosaic technique to investigate whether the muscle degeneration of the *aperC*^{TF36} mutation is of myogenic or neurogenic origin. The focus for the PER defect mapped on the blastoderm fate map suggests that the nervous system is the primary site of the cause of muscle degeneration.

MATERIALS AND METHODS

Flies

Fruit flies, *Drosophila melanogaster,* were reared on the usual cornmeal-yeast medium under constant illumination. The Canton-S strain was used as a wild-type. The *aperC*^{TF36} mutation isolated in our laboratory is recessive and is mapped at 0.4 of the X chromosome [12]. The wild-type, *aperC*^{TF36} mutant and mosaic flies were reared at 25°C until eclosion and kept at 20°C after eclosion. The *aperC*^{TF36} mutation is temperature sensitive [3]. The sensitive period is 2–3 days after eclosion. At 20°C, the loss of PER is almost complete, nor does PER recover at advanced stages.

Production of genetic mosaics

Gynandromorphs were produced by using the unstable ring-X chromosome, *In(1)w*^{vC}. The *aperC*^{TF36} mutant chromosome was marked with four recessive genes for surface structure. Mutant males of *y aperC*^{TF36} *w sn*^{3} *f*^{36a}/*B*^{s}Y were crossed to females of *In(1)w*^{vC}/*y w spl* (for genetic symbols, see Lindsley and Grell [14]). Loss of the ring-X chromosome from a part of nuclei of the *In(1) w*^{vC}/*y aperC*^{TF36} *w sn*^{3} *f*^{36a} female zygotes at or after the first mitosis results in the genetic mosaics [11]. The mutant phenotype is expressed in the cell lineage with the male genotype (*y aperC*^{TF36} *w sn*^{3} *f*^{36a}/0) caused by a loss of the ring-X chromosome. The mutant phenotype is masked in the cell lineage with the female genotype (*In(1) w*^{vC}/*y aperC*^{TF36} *w sn*^{3} *f*^{36a}). Cell lineages with the male genotype produce a white eye and yellow cuticle with singed-forked bristles. Those with the female genotype produce a variegated eye and wild-type cuticle and bristles.

Examination of the PER defect

Flies were starved for about 20 hr with free access to water. About 5 hr prior to the test, the flies were anesthetized by chilling. The mesonotum and wings were pasted on acryl board, ventral side up, with myristyl alcohol melted by a needle-shaped heater. All legs and proboscis were free to move. The fixed flies were kept in a moist chamber. A small hemispherule of test solution ejected on the tip of a hypodermic needle from a 1-ml plastic syringe was brought into contact with the prothoracic tarsi or proboscis labella. The flies were first satiated with distilled water. The PER was examined with 1 M sucrose solution.

Examination of the muscle degeneration

After the examination of the PER defect, the degeneration of the rostral protractors was observed by polarizing light microscopy. Flies were fixed in 70% alcohol, dehydrated in graded alcohol series, and cleared in methyl benzoate. The head was separated from the body and then mounted in Bioleit (Oken Shoji Co.). The preparations were viewed in a frontal plane under a polarizing light microscope or a dissecting microscope equipped with crossed-polarizers. The axis of the analyzer was perpendicularly crossed with that of polarizer. The midline of the fly's head was aligned along the bisector of the angle between polarizer and analyzer. Because of the birefringence of the contractile elements, the normal rostral protractors were seen brightly and the brightness was equal on both sides. If the muscles degenerated, the brightness weakened or disappeared (Fig. 1).

Mapping of the behavioral focus

Prior to the examination of the PER defect, each mosaic fly was immobilized by ice-chilling and the genotypes of the surface structure (landmarks) were scored according to the color of cuticle or eyes and the form of the bristles. If both the mutant and the wild-type cuticles were mixed

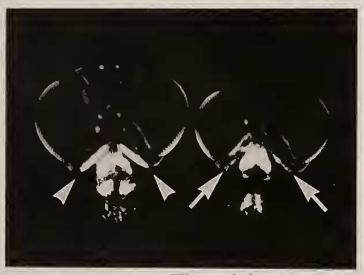

FIG. 1. Heads of *y aperC*^{TF36} *w sn*^{3} *f*^{36a} male in whole mount preparation under crossed polarizers. Left: 1 day after eclosion; arrowheads: rostral protractors with normal birefringence. Right: 5 days after eclosion; arrows: weakened birefringence of the muscles. Both at 25°C.

within a large structure, it was counted half as normal and half as mutant. The correlations between the genotypes of surface landmarks and the PER phenotypes were summarized into matrices.

The distance between two external structures on the blastoderm fate map is represented by the probability that the mosaic boundary passes between them. The unit of the distance on the blastoderm fate map is called a "sturt" [11, 15]. One sturt means that the genotypes of a pair of structures differ from one another in 1% of all the mosaics observed. As well as the distance between the two external sites, the distance between an external landmark and a behavioral focus can be calculated [11]. Distances between the focus for the PER defect and various surface landmarks were calculated. The submissive focus model in which the effect of a hemilateral mutant focus is masked by the wild-type focus on the other side of the mosaic fly [11] was applied. The focus for the PER defect was located on the blastoderm fate maps of Hotta and Benzer [11] and of Koana and Hotta [16].

RESULTS

Correlation between PER defect and muscle degeneration in the aperC^{TF36} *mutant*

The PER to the sugar stimulus and the birefringence of the rostral protractors examined in 50 wild-type flies were normal irrespective of ages.

The PER phenotypes of the *aperC*^{TF36} mutants were examined at various ages after eclosion. The mutants came to show a defective PER at advanced ages (Fig. 2). At day 3 of eclosion, all mutants extended the proboscis normally to the sugar stimulus. At day 5 about 60% of the mutants showed the PER defect. Later, most of the mutants showed the PER defect. By day 14, only a few had recovered the PER.

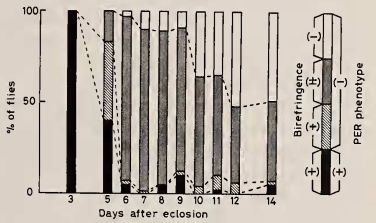

FIG. 2. Progression of the PER defect and the loss of birefringence of rostral protractors at 20°C. The column on the right indicates the category of the phenotypes. About 50 flies were examined for each column.

After the PER test, the birefringence of the rostral protractors of the mutant flies was examined (Fig. 2). All mutants which showed normal PER possessed the normal muscular birefringence. The degree of birefringence varied in the mutants with abnormal PER. The degree of birefringence was classified into three groups according to its brightness under the crossed polarizers; (+): brightness is similar to that of the wild-type fly, (−): brightness is absent or patchy if present, and (±): brightness is present but markedly weak compared with that of the wild-type fly. At day 5, about 67% of the PER defective mutants still showed normal birefringence (+). After that, the number of mutants with normal birefringence rapidly decreased, whereas the number of flies with no birefringence (−) increased. After day 6 of eclosion, the loss of the birefringence of the mutant's rostral protractors correlated with the PER defect. Most of the PER defective flies

showed abnormal birefringence (− or ±). Only a small percent of the PER defective flies showed normal birefringence (+). No differences were seen in the birefringence between the bilateral pair of the muscles.

Correlation between PER defect and muscle degeneration in the mosaic flies

The PER phenotypes of the mosaic flies correlated with the birefringences of the rostral protractors (Table 1). The PER phenotype and the birefringence were examined at 9–11 days after eclosion at 20°C. The mosaic flies showed either normal (+), weak (±) or no (−) birefringence in the rostral protractors. Weak (±) or no (−) birefringence were counted as abnormal. The mosaics with normal PER showed normal birefringence, whereas the mosaics with defective PER showed abnormal birefringence, although the correlation was not absolute. Of 207 mosaic flies, 144 showed normal PER, 62 showed defective PER and one fly extended the proboscis only on the right side of the body. Of 144 PER normal flies, 135 ones showed normal birefringence (+) and only 9 showed abnormal birefringence. Out of 62 PER defective flies, only 3 demonstrated normal birefringence. The fly with the right-sided PER showed normal birefringence on the left side rostral protractor but no birefringence on its right side protractor. No other left-right asymmetries were observed in the PER defect or in the birefringence.

The head surfaces of 89 mosaics were solidly of either wild-type or mutant (Table 2A). The genotype of the head surface coincided with the PER phenotype in most mosaics. Of 46 mosaics whose head surface was entirely wild-type, all flies showed normal PER. Of 43 mosaics whose head surface was entirely mutant, 36 flies showed defective PER and 7 flies showed normal PER. The muscle birefringence also correlated with the genotype of head surface (Table 2B). Forty five of the 46 mosaics with the wild-type head surface showed normal birefringence and only one of them showed abnormal birefringence. Thirty seven mosaics out of 43 with the mutant head surface showed abnormal birefringence and 6 of them showed normal birefringence.

All the 14 mosaics whose head had a mosaic

TABLE 1. Correlation matrix between the PER phenotype and birefringence of the rostral protractors, in the *aperC*^{TF36} mosaics, 9–11 days after eclosion, 20°C

		PER phenotype	
		normal (wild)	abnormal (mutant)
Birefringence	normal (wild)	135	3
	abnormal (mutant)	9	59

TABLE 2. Correlation matrices between head surface genotype and the PER phenotype (A) and the muscle birefringence (B) in the *aperc*^{TF36} mosaics

(A)

		Entire head cuticle	
		wild-type	mutant
PER Phenotype	normal (wild)	46	7
	abnormal (mutant)	0	36

(B)

		Entire head cuticle	
		wild-type	mutant
Birefrin-gence	normal (wild)	45	6
	abnormal (mutant)	1	37

TABLE 3. Summary of focus mapping of the PER defect: Correlation matrices for 12 surface landmarks and fate map distances ($\overline{Af}$)

	Correlation matrices*			Calculated distances in sturts#
Surface landmarks[1]	a_{11} b_{11}	a_{10} b_{10}	a_{00} b_{00}	$\overline{Af}$
ANT	147 2.5	62.5 21.5	8.5 49	13.2
OC	151 2	54 15	13 56	12.8
PV	159 6	46 17	13 50	15.3
OV	153 9	48 19	17 45	19.2
PA	138 2	71 26	9 45	15.0
IV	153 5	50 20	15 48	17.1
PR	146.5 20.5	41 28.5	30.5 24	35.8
HU	127.5 23	63 28	27.5 22	33.9
ANP	97 7	97 46	24 20	31.4
ADC	110 15	78 39	30 19	35.3
LEG1	126 13	77 32	15 28	24.8
4t	122.5 41	70.5 25	25 7	43.3

[1]abbreviations for surface landmarks: ANT, antenna; OC, ocellar bristle; PV, post vertical bristle; OV, outer vertical bristle; PA, palp; IV, inner vertical bristle; PR, proboscis; HU, humeral bristle; ANP, anterior notopleural bristle; ADC, anterior dorsocentral bristle; LEG1, foreleg; 4t, 4th abdominal tergite.

*a_{ij} and b_{kl} represent the number of flies classified in each category.

		A pair of surface landmarks, A and A′		
		Both wild-type	One wild one mutant	Both mutant
PER phenotype	normal	a_{11}	a_{10}	a_{00}
	abnormal	b_{11}	b_{10}	b_{00}

#Map distance $\overline{Af}$ between the surface landmark (A) and the ipsilateral behavioral focus (f) is calculated according to the submissive focus model [11].

$$\overline{Af}=(1-\overline{AA'})\times(a_{00}/(a_{00}+b_{00})+b_{11}/(a_{11}+b_{11}))/2 + \overline{AA'}\times b_{10}/(a_{10}+b_{10}),$$

where $\overline{AA'}=(a_{10}+b_{10})$/total number of mosaic flies.

boundary at the midline showed normal PER and normal birefringence on both sides of the rostral protractors.

Mapping the focus for the PER defect

The PER phenotype and the genotype of 12 pairs of surface landmarks were examined in 291 mosaic flies (Table 3). The PER of the mosaic fly was examined individually 7 days after eclosion, so as to minimize any error due to the incomplete penetrance of the mutation (data not shown).

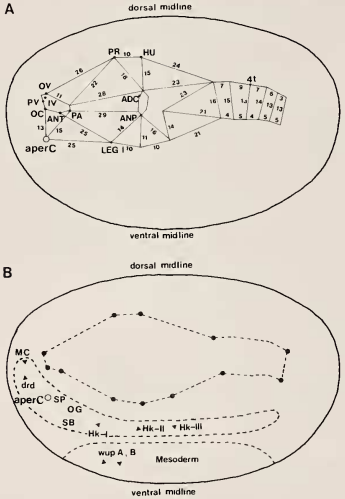

Fig. 3. A: the defective PER focus (open circle, aperC) of the *aperC*^{TF36} mutation on the blastoderm fate map redrawn from Hotta and Benzer [11]. For abbreviations of surface landmarks, see Table 3. B: the *aperC* focus on the fate map redrawn from Koana and Hotta [16]; the presumptive regions of the central nervous system and the mesodermal structures are inferred and shown with dotted line. Abbreviations: drd§, *drop-dead* focus; Hk-I, II, III§, *Hyperkinetic* leg shaking foci for pro-, meso-, and metathoracic legs respectively; MC†, male courtship focus; OG*, optic ganglion; SB*, subesophageal ganglion; SP*, supraesophageal ganglion; wup-A, B§, foci for two *wing-up* mutants. §from Hotta and Benzer [11], †from Hotta and Benzer [23], *from Kankel and Hall [17].

Seventy three flies showed the mutant phenotype in PER and 218 flies showed normal PER. The one fly with the right-sided PER was counted as a mutant. The correlation matrices between the genotypes of various surface landmarks and the PER phenotype of an individual suggest that the focus is located in the anterior region of the animal. The distances ($\overline{Af}$) on the fate map between the PER focus (f) and a surface landmark (A) were calculated according to the submissive focus model [11]. The triangulate mapping located the focus for the PER defect in the antero-ventral region of the blastoderm fate map (Fig. 3).

DISCUSSION

Amongst our mosaic flies, the PER defect correlated with the loss of birefringence of the rostral protractors. The same correlation has been seen during the course of progression of the PER defect in the *aperC*^{TF36} mutant (Fig. 2 and [3]). These correlations indicate that the PER defect of the *aperC*^{TF36} mutation is caused by the degeneration of the muscles, or that the PER defect and the muscle degeneration result from a common but non-muscular cause. The behavioral focus for the PER defect and the focus for the muscle degeneration must be located on the same site of the blastoderm fate map. The focus of the PER defect suggests the site where the *aperC*^{TF36} mutation exerts its primary effect responsible for the muscle degeneration.

The phenotypes of the *aperC*^{TF36} (the PER defect and the muscle degeneration) closely correlated with the genotypes of the head cuticle in the mosaics whose entire head cuticle shows a solid genotype. However, there were several exceptional cases in which the genotype of head cuticle and the PER or muscle phenotype did not match. This indicates that the area of blastoderm which gives rise to the defect of the *aperC*^{TF36} mutation is close to, but distinct from the area which gives rise to the head cuticle.

Very few mosaics showed any hemilateral abnormality. The mosaic flies were either wild- or mutant type for the PER except for two cases out of 498. No other intermediate PER abnormality was found. Birefringence of the muscles in the

mosaics was also either wild- or mutant type with bilateral symmetry except for one out of 207. The phenotypic expression of the *aperC*^{TF36} mutation seems therefore to be an all or nothing event in an animal as a whole.

Homolateral expression in the mosaics led us to consider three possibilities for the focus which causes the defect of the *aperC*^{TF36} mutation: a single focus, two bilateral independent foci and two bilateral interacting foci. If the *aperC*^{TF36} defect is expressed by a single focus, the genotype of the focus is either mutant or wild-type with equal probability, and half the mosaic flies studied would show the mutant phenotype. Since 70% of the mosaics were wild-type in the PER and muscle birefringence, however, no single focus model is applicable. If two bilateral foci cause the defect independently on each side of the body, hemilateral abnormality should appear in proportion to the distance between the foci. The very low frequency of hemilateral abnormality may indicate that a pair of bilateral foci are independent but very close to the blastoderm midline. As this would be equivalent to a single unpaired focus on the midline, we can rule out the possibility of bilateral independent foci as well, since we found a much larger frequency of mosaics with the wild-type than those with the mutant phenotype. This is reinforced by the result that none of the mosaics whose head surface was separated into the wild-type and the mutant at the midline showed hemilateral abnormality. It is therefore plausible to suggest that the bilateral foci are interacting together to express a single phenotype in a fly as a whole.

When two bilaterally homologous PER foci are different in genotype, the interaction of both the mutant and the wild-type focus must result in either a to tally mutant or a totally wild-type fly. The resultant phenotype depends on whether the mutant focus is submissive to or domineers over the wild-type focus [11]. Because the majority of the mosaic flies were wild-type, the *aperC*^{TF36} mutation focus is assumed to be submissive to the wild-type focus. The mutant phenotype results only when both of the bilateral foci are mutant.

Location of the *aperC*^{TF36} focus obtained from the submissive focus model suggests that the primary cause of the muscle degeneration lies in the nervous system. The focus of the *aperC*^{TF36} was located in the ventro-anterior region of the blastoderm fate map. This region of the fate map corresponds to the brain of the adult fly [17]. A possible explanation for the submissiveness is that the normal side of the brain supplies a sufficient amount of a factor, e.g. of neuro-endocrine nature, which controls the posteclosional maintenance or maturation of the muscles.

It is unlikely that the muscle degeneration of the *aperC*^{TF36} mutation is myogenic. If the muscles with a mutant genotype degenerate of themselves, the hemilateral degeneration would appear frequently in the mosaics. We can find this case in the *heldup*^{2} (*hdp*^{2}) mutation, which causes a degeneration of flight muscles in the late pupal development. Quite a large proportion of the mosaics of the *hdp*^{2} mutation have shown such hemilateral muscle degeneration [18]. The primary focus for *hdp*^{2} degeneration was mapped on the ventral mesoderm of the presumptive musculature [18]. In contrast to this, hemilateral abnormality appeared very rarely in the *aperC*^{TF36} mosaics.

The homolateral expression in the mosaic may indicate a bilateral origin of the muscle: migration of precursor cells across the midline. As our present methods of examination of the birefringence and the PER may not be sensitive enough to detect the loss of half amount of muscles, the majority of the mosaics may have been allotted as normal although suffering from some myogenic degeneration. Cell lineage analysis by the mitotic recombination and a histochemical marker [19] implies that the rostral protractor is of unilateral origin, whereas many of the other head muscles are of bilateral origin.

We can not, of course, rule out the possibility that the primary focus of the *aperC*^{TF36} mutation is in the muscles per se, because location of a focus by triangulation in the anterior portion of the blastoderm is sensitive to statistical errors [20]. To obtain direct evidence, we must use histochemical markers to examine the genotypes of muscles [19, 21, 22] or of nervous tissues [17].

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